Transport of *Rhizobium* and *Pseudomonas* through Soil[1]

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ABSTRACT

A study was made of the vertical movement of *Rhizobium japonicum* and *Pseudomonas putida* added to the surface 2.4 cm of nonsterile soil. Recovery of low numbers of these bacteria was possible because the strains were resistant to combinations of inhibitors that prevented growth of most soil microorganisms on agar media. Viable cells of the two bacteria were not transported below 2.7 cm in moist soil in the absence of some transporting agent or in the presence of developing roots of soybeans (*Glycine max* (L.) Merr.) or beans (*Phaseolus vulgaris* L.). Percolating water and a burrowing earthworm (*Lumbricus rubellus*) enhanced the vertical transport of *P. putida* but had a smaller effect on *R. japonicum*. Dispersal of both bacteria was more extensive in the presence of percolating water together with *L. rubellus* or developing roots. *Pseudomonas putida* was transported farther and in greater numbers than *R. japonicum*. However, <1 and 4% of the recovered viable cells of *R. japonicum* and *P. putida*, respectively, were present below 2.7 cm.

Additional Index Words: **dispersal, earthworms, percolation.**

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When nodulation of legumes takes place as a result of seed inoculation, dispersal of rhizobia must have occurred because the bacteria must migrate or be transported from the seed coat to the infection foci on the developing root. Hence, the dispersal of the root-nodule bacteria is of considerable importance for successful inoculation.

Several investigators have studied the transport of *Rhizobium* in soil. Brockwell and Whalley (1970) suggested that nodulation may be limited by the migration of the inoculum to the infection sites on the seedling root. Nobbe et al. (1892) found that plants grown in sand inoculated with rhizobia at a depth of 20 cm bore nodules only on deeper roots, and they concluded that rhizobia are unlikely to move readily through soil. Kellerman and Fawcett (1907) observed that *Rhizobium leguminosarum* moved laterally about 2.5 cm in 48 hours in sterilized soils saturated with water. Frazier and Fred (1922) found that nodules appeared on roots that had grown within 17 cm of a *Rhizobium* inoculum placed at a fixed site in sterilized soil. Chatel et al. (1968) reported that *R. trifolii* moved 5.0 cm laterally from an inoculated row of *Trifolium subterraneum* to nodulate uninoculated plants. Robson and Loneragan (1970) suggested that wind and water may have been responsible for the lateral dispersal of *R. meliloti* for a distance of 5 cm in the field, and Brockwell et al. (1972) attributed the dispersal of *R. trifolii* between plots to the lateral movement of rainwater through and over the soil. Based on studies with sterile soil, Hamdi (1974) concluded that the downward movement of an inoculum of *R. trifolii* was proportional to the quantity of water applied to the soil surface. Thus, no clear picture exists on the extent of transport.

Despite the significance of dispersal of rhizobia, knowledge of their transport is limited, and much of the data is from uncontrolled field experiments, is qualitative, or has been obtained in studies of autoclaved soil, in which growth may occur and biotic stresses on *Rhizobium* have been eliminated. Hence, a quantitative study was conducted using nonsterile soil to assess the significance of several means of transporting rhizobia. In addition, the behavior of a strain of *Pseudomonas* was also investigated to provide data on the dispersal of a common genus in the bacterial community of soil.

MATERIALS AND METHODS

A mutant of *R. japonicum* 3I1b138 that fixed nitrogen on soybeans and was resistant to 1.0 mg of streptomycin sulfate and 60 μg of erythromycin/ml and a strain of *P. putida* that grew in an agar medium containing 1.0 mg of streptomycin sulfate, 50 μg of erythromycin, and 75 μg of thiram/ml were used. The bacteria were grown in a medium containing: K_2HPO_4, 0.50 g; $MgSO_4 \cdot 7H_2O$, 0.20 g; NaCl, 0.10 g; mannitol, 10 g; yeast extract (Difco Laboratories, Detroit, Mich.), 1.0 g; and distilled water, 1.0 liter (YEMB). The cells used for inoculating soil were grown in this medium at 30°C on a rotary shaker operating at 120 rpm; after 6 days for *R. japonicum* or 1 day for *P. putida*, the suspension was diluted in a sterile solution of the salts used in the growth medium to about 10^7 cells/ml.

Freshly obtained *L. rubellus* was grown at 20 to 22°C in Collamer silt loam (Glossoboric Hapludalf) amended with approximately 0.1% each of grass clippings and maple-leaf litter and water to 20% (wt/wt). The worms were 7 to 10 cm in length, active, and free of visible injury.

Samples of Honeoye gravelly silt loam (Glossoboric Hapludalf, pH 7.3), which was used in all experiments, were collected from the 0 to 40-cm depth and sieved while moist (14 to 16% water) through a 2-mm mesh wire screen immediately before each experiment. Sieved soil was added to polyvinyl chloride cylinders (10 cm i.d., 10 cm in height) that were sealed at the bottom with two layers of polyethylene containing a no. 7 rubber stopper (2.4 cm in height), large end downward, at the center of the polyethylene sheets. The soil was tamped until the cylinder was full, and a bulk density of 1.1 g/cm^3 resulted. The top of the cylinder was closed with a double layer of polyethylene, and the cylinder was then inverted, no mixing of soil occurring because the cylinder was full. The polyethylene and rubber stopper were carefully removed from the upper end of the cylinder, and the remaining cavity was filled to its 2.4-cm depth with 27 g of Honeoye soil inoculated with about 10^6 cells of *R. japonicum* or *P. putida* per gram. The inoculated soil, which had the same moisture content as that in the cylinder, was mixed while moist and sieved through a 2-mm mesh wire screen prior to being added. Sieving was omitted in a study of the effect of unreduced aggregate size on movement. Some of the soil columns received worms or seeds, and then the cylinders were covered with a double layer of polyethylene and incubated for 7 days.

Seeds of *Phaseolus vulgaris* L. var. Red Kloud and *Glycine max* (L.) Merr. var. Evans were moistened for 12 hours

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and then planted in the center of the inoculated soil at a depth of 0.5 cm. The polyethylene covers on the planted cylinders were sliced on day 3 to allow the plants to emerge. By day 7, the seedlings were 8- to 18-cm tall, and the primary leaves were fully opened. The roots had extended downward 9.5 cm to the lower end of the cylinders.

The open end of 18-ml test tubes containing individual worms was placed in the center and 4 mm below the surface of the inoculated soil. Within 15 minutes, the worms had burrowed into the soil. Casts and channels were regularly found when experiments were terminated, indicating that burrowing had occurred.

About 3 hours before removing the cores on day 7, water equivalent to 0.5 or 1.0 cm of rainfall was applied for about 10 minutes with a hand-operated misting device (Trigger Spray, Science Products Co., Chicago). The quantity and rate of application of water were not sufficient to result in leaching or puddling. Before spraying water on the soil surface, a conical container (9 cm at the base), the bottom of which had been removed, was placed on top of the soil. The walls of the container guided water onto the soil and thus minimized the flow of water along the inner surface of the cylinder.

All cylinders of soil were incubated for 7 days. During that period, the cylinders containing plants were exposed to 14 hours of light at 28°C and 10 hours of darkness at 20°C. All other cylinders were exposed to 11 hours of light at 16°C and 13 hours of darkness at 10°C. Lighting was by cool white fluorescent lamps (200 to 250 μEinstein sec^{-1} m^{-2}). All treatments were in quadruplicate unless otherwise noted.

At the end of the incubation period, the cylinders were inverted, and the polyethylene at the top was removed. No mixing of soil occurred because the cylinder was full. A sterile brass pipe (14.5 cm in length, 2.4 cm i.d., and 2.6 cm o.d.) with a sharpened, beveled edge was forced slowly into the soil. The pipe was slowly rotated to reduce compaction of the resultant core, which was taken from the center of the cylinder. This core included taproots (when plants were present) and a section of the original inoculated soil. A sterile bottle-cap, 2.4 cm in diameter, was forced into the lower end of the brass pipe, where the cap abutted the inoculated soil. The soil core was then forced through the pipe by placing pressure on the cap. As the soil core became exposed, it was cut into five segments with a spatula that was sterilized before each segment was removed. Because the incubated portion of the soil column entered the brass pipe last and moved through the pipe and was extruded last, contamination of the lower layers of the soil by the upper layers did not occur. The compaction occurring during the removal of the core was estimated from the ratio of core heights before and after core removal. It was assumed that compaction was uniform in the core. Segments representing depths of 0 to 2.7, 2.8 to 4.2, 4.3 to 5.7, 5.8 to 7.2, and 7.3 to 10.0 cm of the originally uncompacted soil core were thus obtained. A single core was removed from each cylinder.

The segments were suspended and diluted in sterile salts solution, and *R. japonicum* was counted using pour plates of YEMB agar containing 1.0 mg of streptomycin sulfate, 60 μg of erythromycin, 400 μg of cycloheximide, 200 μg of nystatin, and 25 μg of congo red/ml. *Pseudomonas putida* was counted using spread plates of YEMB agar containing 1.0 mg of streptomycin sulfate, 50 μg of erythromycin, 125 μg of cycloheximide, and 75 μg of thiram/ml. The plates were incubated at 30°C for 8 days for *R. japonicum* and 2 days for *P. putida*.

Thiram (tetramethylthiuram disulfide, 97%) was from Aldrich Chemical Co., Milwaukee, Wis. Cycloheximide, nystatin, streptomycin, and erythromycin were from Sigma Chemical Co., St. Louis, Mo. Congo red was from Allied Chemical and Dye Corp., New York, N.Y. All of the antibiotics were sterilized by filtration except nystatin, which was not sterilized but was added either as a powder or in a methanol suspension to media shortly before pouring; media prepared in this manner and incubated at 30°C for 10 days showed no contamination.

RESULTS

Rhizobium japonicum and *P. putida* maintained their antibiotic resistance when grown in antibiotic-free YEMB for more than 20 generation times. Thus, it is unlikely that reversion to the wild type occurred during the experiments. In tests of the ability of the test organisms to survive in nonsterile soil maintained at 20°C and 15% moisture (wt/wt), no change in *R. japonicum* population size was found in 7 days, which was the only sampling time, when the inoculum was about 10^3 cells/g. The numbers of *P. putida* increased from 630 to 1,700/g in the same period. Hence, the bacteria appear to survive in soil, thus allowing for measurement of their transport by counting viable cells.

The medium used to count *P. putida* was highly selective for this bacterium; thus, when 1.0-ml portions of a suspension of 1.0 g of soil in 3.0 ml of the salts solution was plated, only the smooth, beige colonies of *P. putida* appeared on the agar surface after 2 days of incubation. As few as 3 *P. putida* cells/g could be detected. On the other hand, the medium used to count *R. japonicum* was not as selective, and agar inoculated with 1.0-ml portions of a suspension of 1.0 g of soil in 30 ml of the salts solution had much of its surface covered by fungal and bacterial growth; in these instances, plates at the next dilution were counted, but these plates often contained few colonies

Table 1—Number of *R. japonicum* at various depths in inoculated soil in the presence of plants, *L. rubellus*, or 0.5 cm of percolating water.

	No. × 10^3/g of soil						
Depth, cm	Untreated	*G. max*	*P. vulgaris*	*L. rubellus*†	0.5 cm water†	*G. max* + water	*L. rubellus* + water
0.0–2.7	410 ± 140‡	520 ± 140	490 ± 210	430 ± 80	410 ± 57	600 ± 120	300 ± 150
2.8–4.2	0	0	0	0.005 ± 0.01	0.1 ± 0.3	6.5 ± 2.4	1.0 ± 1.0
4.3–5.7	0	0	0	0.05 ± 0.1	0	1.3 ± 1.1	0.4 ± 0.7§
5.8–7.2	0	0	0	0.05 ± 0.1	0	0.01 ± 0.03	0.3 ± 0.6§
7.3–10.0	0	0	0	0.008 ± 0.02	0	0	0

† Where values other than zero are shown at depths below 2.7 cm, 3 of the 4 replicates did not contain detectable *R. japonicum*, and the plates from the lowest countable dilution from the fourth replicate contained 10 or fewer colonies.

‡ Mean ± SD of 4 replicates, except for 3 replicates at top two depths in *G. max* + water treatment.

§ No detectable *R. japonicum* in 1 replicate at 4.3- to 5.7-cm depth and none in 2 replicates at 5.8- to 7.2-cm depth. Counts based on < 23 colonies per plate at the lowest countable dilution.

of *R. japonicum*. By these methods, as few as 30 *R. japonicum* cells/g could be detected.

The results of a study of the possible movement of *R. japonicum* through soil in the presence of growing plant roots, *L. rubellus*, or percolating water are given in Table 1. In the absence of a worm, plant, or percolating water, movement of viable cells below the 2.7-cm depth was not detected. Roots of *G. max* and *P. vulgaris* growing through the inoculated zone of soil in the absence of percolating water failed to transport detectable numbers of *R. japonicum* below the surface layer. The counts in soils containing *L. rubellus* were always low in the absence of percolating water. Percolating water alone transported a small number of viable *R. japonicum* cells into the layer immediately underlying the inoculum, but not beyond 4.2 cm. In contrast, when soil received 0.5 cm of percolating water and contained growing roots or *L. rubellus*, *R. japonicum* was transported to a depth between 5.8 and 7.2 cm. The percentages of viable *R. japonicum* cells recovered at the end of the experiment that were present below 2.7 cm were 0.013, 0.013, 0.65, and 0.35 for cylinders receiving *L. rubellus*, 0.5 cm of percolating water, *G. max* plus water, and *L. rubellus* plus water, respectively.

The results of a study of the movement of *P. putida* are given in Table 2. In the absence of a vector or percolating water, movement below 2.7 cm was not detected. Extension of roots of *G. max* and *P. vulgaris* through the inoculated zone failed to transport detectable numbers of *P. putida* below 2.7 cm. The counts in soil containing *L. rubellus* were variable but always low in the absence of percolating water. On the other hand, the percolation of 0.5 or 1.0 cm of water transported *P. putida* throughout the soil column. The transport of *P. putida* occurred to a depth between 7.3 and 10.0 cm in soil containing *L. rubellus* and receiving 0.5 or 1.0 cm of percolating water. The proportion of the *P. putida* population found at depth in columns percolated by 0.5 cm of water appeared to be reduced by the presence of *L. rubellus*; however, the results of these two treatments were not significantly different.

The method used to obtain the data in the last two columns of Table 2 was different in that the soil was not sieved, so that the unsieved inoculated soil was chiefly composed of aggregates of approximately 2 mm in diameter, whereas most of the aggregates in the uninoculated, underlying sieved soil were approximately 1 mm in diameter. Under these conditions, *P. vulgaris* enhanced the transport of *P. putida*, an effect that was significant at the 5% significance level. The root of *P. vulgaris* grew straight down the middle of the soil, and the core taken from the center of the soil column sampled the taproot, its rhizosphere, and cells transported along the root channel. The effect of omitting the sieving step in the preparation of inoculated soil is suggested by comparing the results with 0.5 and 1.0 cm of water; thus, the proportion of the total *P. putida* population recovered at depth in the unsieved soil receiving 1.0 cm of water (0.44%) was about one-eighth of the proportion found beneath the sieved soil receiving 0.5 cm of water (3.4%). The percentages of viable *P. putida* cells recovered at the end of the experiment that were present below 2.7 cm were 0.10, 3.4, 1.6, 3.0, 0.44, and 1.8 for cylinders receiving *L. rubellus*, 0.5 cm of percolating water, *L. rubellus* plus 0.5 cm of water, worm plus 1.0 cm of water, 1.0 cm of water alone, and *P. vulgaris* plus 1.0 cm of water, respectively. The recovery figures do not take population increases by multiplication or declines by death into account.

DISCUSSION

Movement of the bacteria through soil in the absence of a transporting agent was not detected. Appreciable movement associated with vertical development of microcolonies or motility is unlikely because, for rhizobia at least, the carbon and energy supply is limiting and growth rarely occurs in the absence of added organic compounds (Chowdhury, 1977; Pena-Cabriales, 1981). Moreover, bacterial growth diminishes with diminishing matric potential and water potential (Stotzky, 1972). Soil is made up of a series of discontinuous surfaces and water films that restrict bacterial motility except under extremely moist conditions (Wallace, 1978). The matric suction of the Honeoye gravelly silt loam (moisture content between 14 and 16%) used in this study was not determined, but based on data compiled by Olson and Dower (1980) for nine New York soils of a textural class similar to Honeoye gravelly silt loam, the matric suction probably was about −10 bars at the water content which prevailed. A very slow rate of dispersal in soil at the suction of the present study agrees with the work of Wong and Griffin (1976), who suggested that

Table 2—Number of *P. putida* at various depths in inoculated soil in the presence of plants, *L. rubellus*, or percolating water.

	No. × 10^3/g of soil								
Depth, cm	Untreated (7)†	*G. max* (3)	*P. vulgaris* (4)	*L. rubellus* (8)	0.5 cm water (4)	*L. rubellus* + 0.5 cm water (4)	*L. rubellus* + 1.0 cm water (4)	1.0 cm water (4)	*P. vulgaris* +1.0 cm water (2)
0.0-2.7	1200±450‡	2000±1100	730±530	1100±470	800±160	960±170	1300±500	1600±500	2000±1200
2.8-4.2	0	0	0	0.64±0.88§	37±12	21±10	34±10	5.1±2.1	23±12
4.3-5.7	0	0	0	0.33±0.62§	10±1.5	6.4±5.0	15±8.0	2.7±1.9	13±9.4
5.8-7.2	0	0	0	0.12±0.35§	4.1±2.2	2.3±2.0¶	11±6.0	2.5±1.6	9.0±4.2
7.3-10.0	0	0	0	0.24±0.45§	0.7±0.6	0.18±0.22¶	8.4±5.9	1.2±0.7	4.3±4.1

† No. of replicates.
‡ Mean ± SD.
§ At depths of 2.8-4.2, 4.3-5.7, 5.8-7.2, and 7.3-10.0 cm, 5, 6, 7, and 5 of the 8 replicates did not contain detectable *P. putida*. In soil samples that contained the bacterium, plates prepared from the lowest countable dilution had 40 or fewer colonies.
¶ Plates prepared from the lowest countable dilution had highly different numbers of colonies but always fewer than 97.

at matric potentials near or below −0.5 bars, bacteria are likely to be confined by water films that envelop the soil particles. In previous studies of active bacterial dispersal, no movement was detected if the soil was at matric potentials near or below −0.5 bars (Griffin and Quail, 1968; Hamdi, 1971), whereas reports of active dispersal in soil probably involved soils at matric potentials above −0.5 bars (Frazier and Fred, 1922; Griffin and Quail, 1968; Hamdi, 1971; Stotzky, 1972; Wong and Griffin, 1976).

According to Wallace (1978), water-facilitated dispersal of bacteria in soil is likely to be limited to only a few centimeters because soil hydraulic conductivity is small relative to short-term motility rates of flagellated cells, and adsorption of bacteria to particles may prevent them from entering freely percolating solution. Bitton et al. (1974) found that the water-mediated movement of *Klebsiella aerogenes* through saturated soil depended on the water content of the soil and the surface properties of the bacterium. The finding that *P. putida* was more susceptible to water-mediated transport than *R. japonicum* agrees with the data of Martin (1971), who reported that when water was applied to saturated soil, fluorescent pseudomonads were selectively partitioned into the leachate.

Within the limits of detection of the enumeration methods used in this study, plant roots alone were ineffective in dispersing the inocula. Although plants stimulate the growth of bacteria situated in close proximity to the roots, the propulsion of the apical meristem and root cap through soil as the root grows does not appear to convey microorganisms. Moreover, Rovira and Campbell (1974) found that root tips were sterile, indicating that they are not serving as dispersal agents. On the other hand, *L. rubellus* was responsible for the downward transport of both test bacteria. Worm-mediated transport of soil microorganisms is in accord with studies of Parle (1963) and Thornton (1970). The differences observed in the extent of transport of *R. japonicum* and *P. putida* may stem from their differing abilities to survive and grow on worm surfaces. The data indicate that dispersal was further promoted by percolating water together with a worm or plant roots, probably because the higher organisms provided channels through which the water could carry the bacteria; these observations are in agreement with those of McCoy and Hagedorn (1979), who reported that bacteria are moved through saturated soils containing channels. The relationship between quantity of percolation water applied and extent of transport that was noted here has also been observed in studies of *R. trifolii* (Hamdi, 1974) and *Phytophthora infestans* (Zan, 1962).

These data indicate that dispersal of microorganisms in soil, which has been actively investigated by public health researchers and plant pathologists, must be considered in developing an understanding of the ecology of indigenous bacteria and of *Rhizobium* strains used as inoculants to enhance nitrogen fixation.

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